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Assembly of the yeast vacuolar H^+-ATPase and ATP hydrolysis occurs in the absence of subunit c″

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Abstract The V-ATPases are ubiquitous enzymes of eukaryotes. They are involved in many cellular processes via their ability to pump protons across biological membranes. They are two domain enzymes comprising an ATP hydrolysing sector and a proton translocating sector. Both sectors are functionally coupled. The proton tanslocating sector, V_0, is comprised of five polypeptides in an as yet undetermined stoichiometry. In V_0 three homologous proteins, subunit c, c′ and c″ have previously been reported to be essential for assembly of the enzyme. However, we report that subunit c″ is not essential for assembly but is for functional coupling of the enzyme.

Keywords: V-ATPase; VMA11; VMA16; Subunit c; Subunit c″

1. Introduction

The vacuolar H^+-ATPases (V-ATPase) is a universal proton pump of eukaryotes. It is a multi-subunit enzyme that is organised into two functional domains, which have specific yet tightly coupled functions [1–3]. A cytosolic sector (V_1) has ATPase activity, and a transmembrane (TM) sector (V_0) is responsible for proton translocation [1–3]. In yeast, the V_1 domain is composed of eight different subunits which form a complex with a molecular mass of ~640 kDa, in a stoichiometry of $A_3B_3C_1D_1E_1F_1G_2H_{1-2}$ [1–3]. V_0 domains in yeast are a complex of five different subunits with a molecular mass of ~240 kDa and a stoichiometry of $a_1, d_1, c_4, c'_1, c''_{1-2}$, with all subunits except subunit d contributing residues important for proton translocation [1–3]. The V_1 and the V_0 domains have been shown by cysteine cross-linking [4] and electron microscopy [5] to be connected via central and peripheral stalks allowing the domains too physically and functionally integrate ATP hydrolysis and proton translocation. The two domains of the V-ATPase interact in a similar way to the F-ATPase, therefore its mechanism of action is believed to be very similar and is thought to operate by rotational catalysis. This is where ATP hydrolysis drives rotation of a central stalk which, in turn causes rotation of the core V_0 domain and allows the translocation of protons across the membrane [6–8].

At the heart of proton translocation in the V-ATPase is a family of conserved highly hydrophobic membrane proteins, the subunit c "proteolipids". In yeast, three forms of proteolipid (c, c′ and c″) are present, and are products of the genes *VMA3*, *VMA11* and *VMA16*, respectively [9,10]. Subunit c (*VMA3*) is a 16 kDa protein arranged as four transmembrane (T.M.) α-helices, and containing an essential DCCD-reactive glutamic acid on the 4th T.M. domain that has been implicated in proton translocation [11]. Subunit c′ (*VMA11*) and subunit c are very similar proteins, and the presence of these two isoforms appears to be a unique specialisation of the fungal enzyme. Subunit c″ (*VMA16*) is a 22 kDa protein which has been shown to be present in all eukaryotes [10]. Secondary structure prediction suggests that subunit c″ is organised as 5 T.M. α-helices, similar to subunit c but with an extra N-terminal helix positioned towards the central pore of the V_0 domain [12]. It shows 25% sequence identity with subunit c and has also been implicated in proton translocation via a glutamic acid on T.M. 3 [12].

Deletion of the genes for any of the three subunit c "proteolipids" in *Saccharomyces cerevisiae*, results in a pH-sensitive conditional lethal (*vma*) phenotype [1–3]. Such cells will grow at pH 5.5 but not at pH 7.5 or in high extracellular Ca^{2+}. In the absence of any one proteolipid species there appears to be no functional or assembled V-ATPase [9,10]. However, we have recently found that in absence of subunit c″, subunit c remains present in the vacuolar membrane and occurs as oligomeric complexes [12]. These are similar to the gap junction-like complexes isolated from vertebrate and arthropod tissues in which these complexes contain only subunit c [13,14].

To resolve this anomaly, we have investigated the nature of the *vma* phenotype that results from the inactivation of the *VMA*16 gene. Far from there being a loss of the V-ATPase, the enzyme is fully assembled in vacuolar membranes and capable of ATP hydrolysis. However, the enzyme is incapable of sustaining a proton gradient, giving rise to the pH-sensitive *vma* phenotype. This indicates that subunit c″ is not essential for maintaining the physical contacts between the V_1 and the V_0 domain, but is required for functional coupling of the two domains.

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Abbreviations: V-ATPase, vacuolar-(H^+)-adenosinetriphosphatase; ATP, adenosine triphosphate; TM, transmembrane; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; NEM, *N*-ethyl maleimide

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2. Materials and methods

2.1. Yeast strains and culture conditions

The following yeast strains were used in this study.

YPH500	*MATα ade2*, *ura3*, *leu2*, *his3*, *trp1*, *lys2*
YPH500 Cl4	As above Δ*VMA16*::*his3*
YPH500 Cl5	As above Δ*VMA16*::*his3*
YPH500 Cl7	As above Δ*VMA16*::*his3*
YPH500 Cl4-1	As above, Δ*VMA16*::*HIS3* Δ*VMA3*::*LEU2*
NUYH1/VMA 3	As above, Δ*VMA3*::*LEU2*
W303-1bvatc	As above, Δ*VMA3*::*LEU2*

The YPH500 strain was a gift of Y. Anraku (Department of Biology, University of Tokyo, Japan). Replacement via homologous recombination of the ORF of each gene with the ORFs of the *S. cerevisiae* HIS3 and LEU2 genes inactivated the *VMA*16 and *VMA*3 genes, respectively. Cultures were maintained on YPD or YNB; the latter supplemented with appropriate nutrients.

2.2. Plasmids

The p424 centromere based plasmid was used for expression of the subunit c″ and subunit c. The inserted gene is under the control of the ADH promoter and is constitutively expressed and the plasmid contains the *TRP2* gene.

2.3. Antibodies

Monoclonal antibodies to the V-ATPase components subunit B (*VMA*2p) were purchased from Cambridge Bioscience Ltd. (24-25 Signet Court, Newmarket Road, Cambridge CB5 8LA).

2.4. Vacuole isolation

Vacuoles were isolated and prepared for ATPase activity measurement as previously described [12].

2.5. ATPase activity

Reactions were performed in duplicate (inhibited and non-inhibited reactions). 10 μg of vacuoles were added to 200 μl of 10 mM Mes/Tris pH 6.9, 5 mM $MgCl_2$, 25 mM KCl and 20μM DTT. To this 0.02% sodium azide was added. To one reaction either 10 μM DCCD or NEM was added and the samples incubated for 5 min. Then 25 μl of 50 mM ATP was added to all reactions and the samples were incubated at 30 °C for 30 min. After incubation the amount of phosphate released was measured by addition of 750 μl of phosphate determination solution (0.5% SDS, 0.5% ammonium molybdate, 0.1% ascorbate, 2% sulphuric acid) and reading of absorbance at 830 nm activity was calculated by the comparison with known phosphate standards.

2.6. Immunoblotting

Proteins were transferred from SDS–PAGE to Hybond nitrocellulose membrane (Amersham Pharmacia) using a semi-dry blotting apparatus. The transfer buffer contained 100 mM Tris, 100 mM glycine, 0.1% SDS, 20% (v/v) methanol. The membranes were blocked with commercial skimmed milk powder in 10 mM Tris/HCl (pH 7.5), 50 mM NaCl and 0.01% (w/v) Tween for 1 h and incubated overnight with the appropriate antibody (1:1000 dilution) and after washing three-times for 10 min in PBS-T the secondary Ab was applied for 1 h (Anti-mouse HRP conjugate, 1 in 2000 dilution). The membrane was then washed 3× 10 min in PBS-T. Bound antibody was detected using ECL detection.

2.7. Cell doubling

50 ml of YPD medium was inoculated to a starting $OD_{600\,nm}$ of 0.01 and grown at 30 °C for 16 h. The optical densities were then converted into cell numbers using colony counting and plotted accordingly, doubling times were also calculated.

2.8. Quinacrine fluorescence

Cells were grown to an O.D. of ~1 overnight in 10 ml of YPD medium. The cells were then cooled on ice for 5 min then 1 ml of the cells were centrifuged and re-suspended in 100 μl of YPD containing 100 mM HEPES pH 7.6 and 200 μM quinicrine, followed by incubation at 30 °C for 10 min. The suspension was cooled on ice for 5 min. Cells were then pelleted and washed twice in 100 mM HEPES pH 7.6 + 2% glucose. The cells were then re-suspended in the aforementioned solution and 4 μl of the suspension was pipetted out onto a microscope slide with 4 μl of 0.5% agarose. This suspension was covered with a coverslip and viewed at 492 nm excitation and 510 nm emission.

3. Results

3.1. Phenotype of the Δvma16 strains

The Cl4 (Δ*vma*16) and Cl4-1 (Δ*vma*3, Δ*vma*16) mutants of the haploid *Saccharomyces cerevisiae*, YPH500 strain have already been described [12]. In addition, we isolated a further two Δ*vma*16 strains from separate rounds of transformations, Cl5 and Cl7, again using the *HIS3* gene as a selectable marker. All four mutant strains exhibited the *vma* phenotype, i.e., growth at pH 5.5 and failure to grow at pH 7.5, or on extracellular 100 mM $CaCl_2$ (Fig. 1). They likewise were incapable of accumulating the pH sensitive dye, quinacrine, into their vacuoles (Fig. 2). They thus appear to have an inactive or unassembled V-ATPase and are incapable of acidifying vacuoles as has been reported by others [9,10].

When the final colony size of wild-type cells, *VMA*16 and *VMA*3 knockout strains were compared they appeared similar, however, *VMA*16 knockout strain colonies grew slower (data not shown). Analysis of the doubling time of Cl4 cells in full (YPD) liquid medium with glucose as the carbon source (Table 1) showed that Cl4 cells grew slower than wild-type YPH500 cells, vatc cells (a *VMA*3 knockout derived from W303 parental strain) or NUY29H1 cells (a *VMA*3 knockout derived from YPH500 parental strain). *VMA*3 knockout strains had a 30% increase in doubling time compared to wild-type but the doubling time of Cl4 was more than twice as long (Table 1).

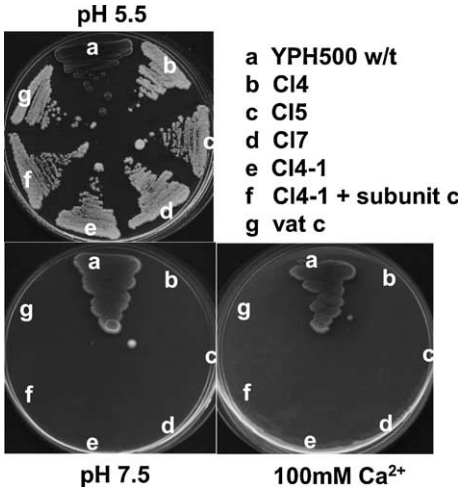

Fig. 1. Growth of the yeast strains (a) YPH500 (w/t), (b) Cl4 (*VMA*16 k.o.), (c) Cl5 (*VMA*16 k.o.), (d) Cl7 (*VMA*16 k.o.), (e) Cl4-1 (*VMA*3 and 16 k.o.), (f) Cl4-1 + subunit c (*VMA*3 and 16 k.o. + *VMA*3AU1 re-constituted) and (g) vat c (*VMA*3 k.o.) on V-ATPase restrictive media (YPD buffered to pH 5.5, 7.5 and 5.5 with addition of 100 mM Ca^{2+}). The cells were grown for 3 days at 30 °C.

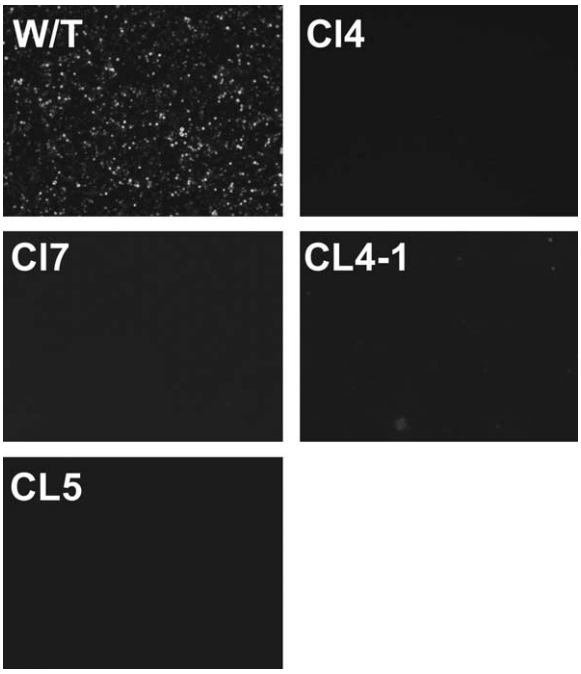

Fig. 2. Quinacrine fluorescence of vacuoles from Cl4, Cl5, Cl7, Cl4-1 and YPH500. Cells were prepared as described in Section 2. Fluorescence was measured at 492 nm excitation and 510 nm emission.

Table 1
Doubling time of yeast knockouts: table showing doubling time of w/t (YPH500), vat c (*VMA*3 k.o.), NUY 29H1/*VMA*3 (*VMA*3 k.o.) and Cl4 (*VMA*16 k.o.). $N = 4$ and times are ±S.E.M.

Strain	Doubling time (h)
YPH500	1.88 ± 0.03
Vat c	2.25 ± 0.01
NUY 29H1/VMA3	2.29 ± 0.05
Cl4	4.36 ± 0.08

3.2. Assembled V-ATPase complexes in the absence of subunit c″

The difference in growth rates between the two knockouts was not expected as our previous studies indicated [12] that subunit c forms oligomeric complexes in the absence of subunit c″. We therefore examined the vacuolar membranes to determine whether there is attachment of V_1 in the knockout strains. Vacuolar membranes were isolated from, wild-type cells, Cl4 cells, Cl5 cells, Cl7 cells, Cl4-1 cells and Cl4-1 cells which had been transfected with a plasmid that encoded subunit c tagged at its C-terminus with the AU1 epitope. These were then immunoblotted for the presence of V-ATPase subunits. Previous studies have shown that if there is no V_0 domain assembly V_1 subunits do not localise to the vacuolar membrane [15].

The results (Fig. 3) demonstrate that the V_1 subunit B is present on vacuolar membranes from w/t cells and is absent in the double knockout strain Cl4-1. However in the absence of subunit c″, Cl4, Cl5 and Cl7 cells, subunit B is detectable at the vacuolar membrane suggesting assembly of the V-ATPase in the absence of this subunit. Also when Cl4-1 cells are transformed with subunit c effectively creating another subunit

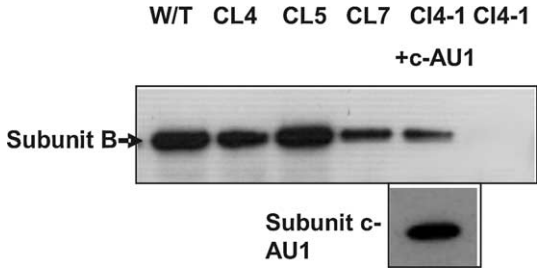

Fig. 3. Western blot analysis of 10 μg of vacuolar fractions isolated from lane (1) YPH500, (2) Cl4, (3) Cl5, (4) Cl7, (5) Cl4-1 + subunit c-AU1 and (6) Cl4-1. Top panel shows blot probed for subunit B and bottom panel is probed for the AU1 epitope.

c″ knockout strain, subunit B and subunit c are detectable at the vacuolar membrane. These results indicate assembly of an enzyme in the absence of subunit c″.

3.3. The presence of an ATPase activity attributable to the V-ATPase at the vacuolar membrane of subunit c″ knockout strains

Immunoblotting of vacuolar membranes indicated the possibility of a non-functional V-ATPase assembled in the absence of subunit c″. Therefore isolated vacuolar membranes were tested for ATPase activity in the presence and absence of the V-ATPase inhibitors DCCD and NEM (Fig. 4A and B).

Nearly 80% of the ATPase activity was inhibited by DCCD in vacuoles isolated from wild-type YPH500 cells (Fig. 4A). As expected, there was only a basal ATPase activity in vacuoles prepared from Cl4-1 cells and the subunit c knockout strain vatc. What little ATPase activity present was largely unaffected by DCCD. However, all three subunit c″ knockout strains had an ATPase activity that was 40–50% of the wild type strain and this activity was inhibited by ~80% upon addition of DCCD. There was a partial restoration of the DCCD-sensitive ATPase activity in vacuolar membranes isolated from Cl4-1 cells expressing the plasmid based subunit c consistent with the re-appearance of subunit B in immunoblots. This was not seen in Cl4-1 cells transformed with subunit c″.

In the presence and absence of NEM the ATPase activity in vacuoles isolated from wild-type cells or from Cl4 or Cl5 cells is reduced to less than 20% by NEM (Fig. 4B). Once again no activity was observed in the Cl4-1 strain, but NEM sensitive activity can be seen on vacuolar membranes from C4-1 cells expressing plasmid based subunit c. This is consistent with the DCCD assay and the re-appearance of subunit B in immunoblots. These data indicate that a V-ATPase capable of hydrolysing ATP but unable to translocate protons is being assembled in the absence of subunit c″.

4. Discussion

Our data shows that the V-ATPase is assembled and capable of ATPase activity in the absence of subunit c″. However, this V-ATPase complex is unable to create a proton gradient across the vacuolar membrane. Therefore subunit c″ is not essential for assembly of the complex but is essential for proton translocation. This data is consistent with earlier studies which have shown that subunit c by itself is capable of forming hexameric

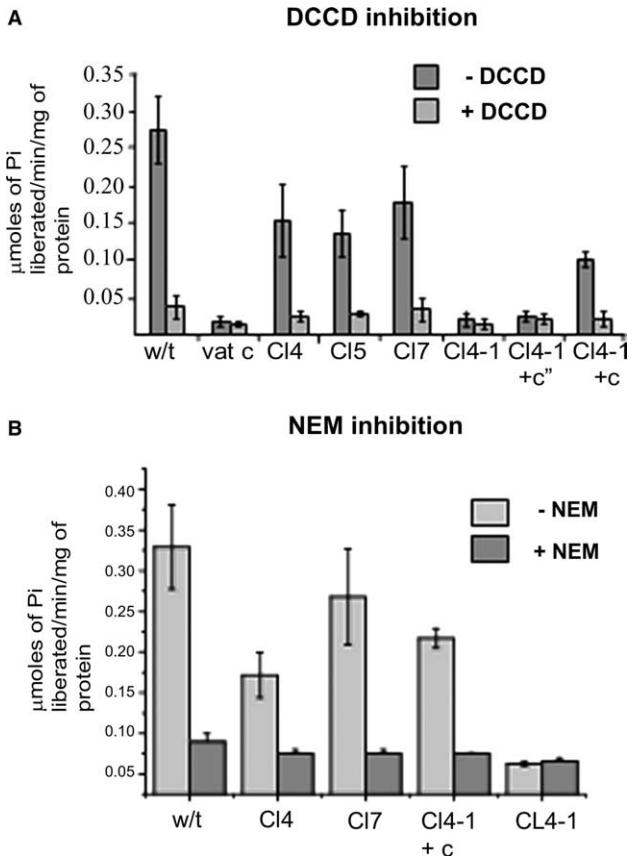

Fig. 4. ATPase activity of 10 µg of isolated vacuolar fractions from w/t, Cl4, Cl5, Cl7, Cl4-1 + subunit c-AU1 and Cl4-1 ($n = 3$, error bars are SEM). (A) Shows assays with (clear bars) and without 10 µM DCCD (grey bars), (B) shows assay with (light gray) and without (dark grey bars) 10 µM NEM. 10 µg of vacuolar membranes were incubated at 30 °C in ATPase activity assay buffer (see Section 2). Activity was determined by incubation with a phosphate detection buffer (see Section 2), and calculated from a standard curve created with known phosphate standards.

complexes in the absence of subunit c″ [12]. However, our results contradict an earlier finding by Hirata et al. [10], which shows that loss of subunit c″ in *S. cerevisiae* results in the absence of the V-ATPase at vacuolar membranes. We have found that in the *VMA* 16 knockout strains, the V-ATPase is labile and it is important to maintain the extracellular glucose concentration, particularly whilst preparing spheroplasts, to maintain the integrity of the V-ATPase. In addition in *S. cerevisiae*, the V-ATPase is known to be sensitive to the nutritional status [16]. Thus, the combination of these two may explain the apparent absence of the V-ATPase activity found by Hirata et al. [10].

The presence of the assembled V-ATPase activity in the absence of subunit c″ raises the question:

Why should the subunit c″ depleted form of the V-ATPase not create/maintain a proton gradient?

It would be predicted from a mechanism based on rotational catalysis that subunit c would be sufficient for forming a proton translocating pathway. This is consistent with a prokaryotic form of the V-ATPase and the F-ATPase [17,18]. The prokaryotic V-ATPase pumps Na^+ instead of H^+, but contains one species of the subunit c family, which is most homologous to subunit c of yeast and does not contain the extra N-terminal located TM domain [17]. Likewise a single of species of subunit c is required for ion translocation in the F-ATPase [19]. Therefore the interaction of individual subunit c protomers with Vph1p, or subunit a, of the V_0 sector could be sufficient for V-ATPase activity. Thus, the machinery for proton translocation would be in place in the absence of subunit c″. However, this is not the case as our results show that the enzyme is assembled at the vacuolar membrane but is unable to establish a proton gradient across the membrane.

One explanation for this may be due to the extra TM domain of subunit c″. This domain has recently been shown to be located toward the centre of the V_0 core [12]. Such a domain could provide a firmer attachment point for the central spindle to prevent slippage between V_0 and the V_1 sector during cycles of ATP hydrolysis. In the absence of subunit c″ this space would be larger and hence may result in failure to rotate the subunit c core complex essential for proton translocation. A recent study by Nishi et al. [20] however has indicated that the N-terminal of subunit c″ is not essential for near full V-ATPase activity. This suggests that the N-terminal helix is not the only attachment point for the rotor stalk and that the other loop regions participate in the attachment of V_1 to V_0.

The formation of an ATP hydrolysing, but non-proton translocating V-ATPase in the absence of subunit c″ would explain the poorer growth rate of these strains. This situation would lead to futile ATP hydrolysis and therefore wastage of energy by the cells as it tries to acidify its vacuole. The cells would then have less energy to devote to other tasks such as growth.

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